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(54) **Nucleic acid amplification employing ligatable hairpin probe and transcription.**

(57) The present invention relates to methods for amplifying nucleic acid sequences. In particular, the invention concerns methods for detecting the presence of a particular nucleic acid sequences with high sensitivity.

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looped structures whose shape resembles a common hairpin. Such hairpin structures are known to occur naturally in many organisms, particularly in RNA secondary structures, however, their functional role is at this point not well established. The physical chemistry of hairpin structures has been described - Cantor and Schimmel, Biophysical Chemistry, Part III, p. 1183, N.H. Freeman & Co. (San Francisco 1980).

The literature on this subject is incomplete and contradictory. For example, there are predictions that hairpins may provide a transcription termination signal - Jendrossek et al, J. Bacteriol. 170:5248 (1988) and Walker et al, Biochem. J. 224:799 (1984). Hairpin structures resembling known rho dependent transcription termination signals have been observed following the unc operon and glms of E. coli. On the other hand, pallindromic sequences capable of forming stable hairpin forms have been found around the transcription initiation site of beta amyloid precursor gene - La Fauci et al, Biochem. Biophys. Res. Commun. 159:297 (1989).

The use of hairpin structures in the synthesis of DNA from oligonucleotides and in the labeling of oligonucleotides is proposed in European Patent Publication 292,802 and by Sriprakash and Hartas, Gene Anal. Techn. 6:29-32 (1989). In addition, Krupp and Soll, FEBS Letters 212:271 (1987) and "Nucleic Acid Probes", ed. Symons (CRC Press, Bacon Raton, FL, 1989) pp. 21 & 22, describe the use of a hairpin structure to make labeled RNA transcripts from an M13 vector/T7 RNA polymerase system.

SUNMARY OF THE INVENTION

The present invention provides a method and means for amplifying a particular nucleic acid sequence of interest (target sequence) by hybridization with and ligation to a probe which renders the target sequence transcribable, and accordingly, capable of providing multiple copies of complementary RNA. The probe has two principal parts, (1) a single stranded self-complementary sequence capable of forming, under suitable hybridizing conditions, a hairpin structure having a functional promoter region, and (2) a single stranded probe sequence extending from, and forning part of the same nucleic acid molecule with, the 3′ end of the self-complementary sequence.

Under suitable hybridizing conditions, the self-complementary region of the probe forms a looped, self-hybridized structure commonly referred to as a hairpin loop, or simply, hairpin. The base sequence of the self-complementary region is selected such that upon formation of the hairpin with the target sequence ligated to the 5′ end of the probe, a desired double stranded promoter sequence is formed operably linked to the ligated target sequence. Thus, the hairpin form of the probe ligated to the target sequence is transcribable in the presence of a suitable RNA polymerase and the required ribonucleoside triphosphates (rNPTs). The sequence of bases in the RNA transcription product will accordingly be complementary with the target sequence.

Transcription can be allowed to proceed for any desired length of time with the accumulated transcription product producing an amplification of the target sequence. Where the target sequence is of analytical interest, high sensitivity detection of the target sequence can be accomplished by amplifying the target in the manner of the present invention and then suitably detecting the accumulated transcription product. Any number of conventional approaches can be taken in order to detect the accumulated RNA transcription product.

For example, the rNPTs added for transcription can comprise a detectable label and, after separating resulting labeled transcription product from the unused labeled rNPTs, the label is detected in the separated product fraction. Another approach is to detect the transcription product by hybridization with a detectable nucleic acid probe and detecting the resulting hybrids in any conventional manner, e.g., using labeled probe or anti-hybrid selective antibody such as anti-DNA/RNA.

Amplification can be further increased by applying a secondary or second stage amplification of the generated RNA transcription product. A variety of methods are appropriate for this purpose, representative examples of which are described in more detail infra.

The present amplification method provides a number of significant advantages over the methods of the prior art. First, the present method requires, in its most general form, but a single probe component as opposed to the need for multiple probes, such as oligoprimers, in many of the prior art methods such as PCR, TAS, and LCR described above. Further, there is no need for time consuming and and cumbersome thermal cycling as with PCR and LCR. Unlike the QβR method described above, the present probe is a simple extended single stranded nucleic acid rather without complex tertiary structure. Other advantages will be evident to the worker in the field.

For T7

```
    TAATACGACTCACTATAG-3'
Tn
    ATTATGCTGAGTGATATC-5'
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For T3

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    ATTAACCCTCACTAAAGGGA-3'
Tn
    TAATTGGGAGTTAGCTTTCC-5'
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For SP6

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    CATACGATTTAGGTGACACTATAG-3'
Tn
    GTATGCTAAATCCACTGTCATATC-5'
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The probe sequence c can be any sequence which is capable of hybridizing with the target sequence of interest. As is known in the art, the degree of homology between the probe and target sequences will depend upon the specific needs of the user. Where a high degree of specificity is needed or desired, perfect or near perfect homology may be required, such in the case of the detection of single base pair mismatches. However, in the normal case, a degree of nonhomology can be tolerated without sacrificing required specificity, amplification, or detection of the target sequence. Thus, the term "complementary" or "hybridizable" will be used herein to describe the desired or necessary degree of homology between the probe sequence and the target. The length of the probe sequence is, in general, not critical. However, in order to obtain rapid and strong hybridization with a selected target sequence, normally probe sequences of at least about 10 bases.

The loop sequence q in the probe which links the self-complementary, hairpin forming sequences a and a′ can be of any composition and length so long as it does not substantially impede or inhibit the formation of the transcribable hairpin or dimer structures. Normally, loop sequence q will be selected to be substantially noncomplementary with itself, e.g., will be composed of a heteropolymeric or homopolymeric DNA or RNA such as a chain comprising only poly T, poly A, poly C, or poly G, or combinations, and will be at least about 2 bases long to allow sufficient steric freedom for loop formation. More usually, loop sequence q will be between about 4 and about 50 bases in length.

The probe is designed such that upon hybridization of a target sequence to probe sequence c, the 5′ terminus of the probe (i.e., the 5′ terminus of either self-complementary sequence a or any intervening sequence b) is ligatable to the 3′ end of the target sequence. In this way, the target sequence becomes operably linked, as is known in the art, to the 5′ end of the promoter. Thus, upon initiation of transcription, the sequences extending from the 5′ end of the promoter are functionally transcribed by the polymerase to form RNA transcripts which comprise a sequence complementary to the target sequence. As indicated above, the probe sequence c can be separated by an intervening sequence b providing that upon ligation of a target sequence to the 5′ end of the probe, corresponding intervening sequence b′ retains operable linkage with the probe sequence, e.g., it does not contain a transcription termination site, is so long as to significantly reduce the efficiency of transcription of the probe sequence, or introduces significant non-specific hybridization. In certain situations it will be desirable to include such an intervening sequence for stability or detection purposes. As mentioned above, where intervening sequences b and b′ are present, it will be generally preferred that they be exactly complementary and thus will hybridize in the hairpin form to position the 5′ end of the probe exactly matching with the opposing 5′ end of the probe sequence. Upon hybridization of the target sequence, the 3′ end thereof becomes efficiently positioned for ligation to the 5′ end of the probe. However, it is also possible that intervening sequence b′ is wholly or partially noncomplementary to intervening sequence b, and may even be of greater length than intervening sequence b. In such case, it is only necessary that intervening sequence b′ be long enough for, and not substantially ninhibitory towards, ligation of the 3′ end of a target sequence that is hybridized to probe sequence c to 5′ end of the probe of the present invention. It has also been found that the efficiency of transcription be dependent upon the sequence presented in the first few (e. g., 3 to 5) nucleotides in the

the mutation such that a DdeI cut fragment will, upon hybridization, align its 3′ end with the 5′ end of the hairpin probe. Such hybridized DNA fragment will be efficiently ligatable to the probe and thereby rendered transcribable. On the other hand, DNA in which the mutation is absent will not be digested although it will hybridize to a substantial extent. However, ligation will proceed much less efficiently because of improper orientation of the ligation partners. Another example using restriction enzymes is the detection of sequences of Chlamydia trachomatis. The sample nucleic acids can be digested with restriction enzymes (SstI to cut the plasmid DNA once or HindIII to cut it several times, Black et al., Current Microbiology (1989) 19:67-74) in such a way that the plasmid DNA of the organism is also digested to produce multiple fragments. Probes corresponding to all such fragments can be hybridized, ligated, and transcribed separately or simultaneously. After transcription, the RNA can be analyzed by hybridization with a single plasmid DNA probe.

DNA can also be degraded by a variety of other means, such as the use of the following types of reagents: EDTA-Fe(II), Stroebel et al. (1988) J. Am. Chem. Soc. 110:7927, and Dervan (1986) Science 232:464; Cu(II)-phenanthroline, Chen and Sigman (1987) Science 237:1197; class IIS restriction enzyme, Rim et al (1988) Science 240:504; hybrid DNAse, Corey et al (1989) Biochem. 28:8277; bleomycin, Umezawa et al (1986) J. Antibiot. (Tokyo) Ser. A, 19:200; neocarzinostatin, Goldberg set al (1981) Second Annual Bristol-Myers Symposium in Cancer Research (Academic Press, New York) p. 163; and methidiumpropyl-EDTA-Fe(II), Hertzberg et al (1982) J. Am .Chem. Soc. 104:313.

The amplification method of the present invention is illustrated in the diagram of Fig. 4. Hybridization of the present hairpin probe and the target sequence present in a sample of nucleic acids produces hybrids (I) in which the probe sequence c in the probe is hybridized with its complementary target sequence c′. In Fig. 4, intervening sequences b and b′ in the probe are depicted as being complementary to one another, which, as discussed herein elsewhere, is not considered critical, but is generally preferred. After hybridization, the juxtaposed 5′ and 3′ ends of probe sequence b′ and target sequence c respectively, are ligated to yield ligated hybrid products (II). With the addition of the polymerase and rNTPs, transcription proceeds with the generation of multiple RNA transcripts having the combined sequence bc.

Transcription is initiated by addition of polymerase and the required rNTPs to the liquid mixture that contains the hybrids comprising the ligated, transcribable target sequence. Under suitable conditions, the synthesis of RNA transcripts will proceed in a continuous manner providing that sufficient amounts of rNTPs are present. Normally, a ribonuclease inhibitor will be included in the transcription reaction mixture in order to avoid undesirable degradation of RNA transcripts by any ribonuclease contamination. Transcription is allowed to proceed for a predetermined period of time or until a detectable or desirable amount of RNA transcript has accumulated. The amount of RNA transcript produced in a given period of time will be proportional to the amount of target sequence present in the original sample. The accumulated transcription product thus serves as an amplification of the target sequence. Transcription can then be terminated by any conventional means such as inactivation of the polymerase or removal of reactants from the mixture.

Further amplification of the RNA transcription products can be accomplished in a number of ways, for example, by the use of replicases such as Qβ replicase or replicase from brome mosaic virus. Also, a separate set of hairpin probe/second probe pairs can be used comprising (1) a transcribable hairpin probe (constructed with a self-annealing, promoter-containing region as in the present probe but with a transcribable probe region complementary with the RNA transcripts extending from the 5′ end, rather than from the 3′ end as in the present probe) and (2) a second probe which hybridizes with an adjacent sequence in the RNA transcription product. After hybridizing the hairpin probe/second probe pairs to transcripts, the hybridized pair is ligated to form transcribable nucleic acids which themselves will produce additional RNA transcript in the presence of the polymerase. Further, the RNA transcripts can be produced to contain a site for immobilization (e.g., by use of ligand, e.g., biotin or hapten, modified rNPTs and immobilization of resulting transcripts by addition of an immobilized form of a ligand binding partner, e.g., avidin or an anti-hapten antibody, respectively), and after being separated from the mixture can be hybridized to a further probe to introduce a promoter site for further transcription. After a few cycles, more than a million-fold amplification is possible.

The following methods in particular are useful for providing a second stage amplification:

(1) Displacing probe from hybrid by RNA - The promoter probe is hybridized with its complementary DNA which is immobilized onto a solid support or hybridized to an immobilizable support. The immobilized or immobilizable support is brought into contact with the product RNA under conditions of specific hybridization. This releases the transcribable probe since RNA will hybridize instead of the DNA because of the stability difference between the RNA-DNA hybrid and DNA-DNA hybrid. After the first stage of transcription, the RNA polymerase activity is destroyed by heating before the mixture is reacted with the immobilized DNA hybrid support under conditions of strand displacement. For every molecule of RNA, one molecule of promoter probe will be produced under the most ideal conditions. By cycling the system it is

methods will be described in detail herein. Principally, these methods are based on the production of labeled transcription product or on hybridization of the transcription product with detection of the resulting hybrids.

(1) Synthesis of labeled RNA transcripts -The addition of rNTPs, one or more of which comprise a detectable label, to the transcription mixture will result in the synthesis of RNA transcripts that also comprise the detectable label. Substances which serve as useful detectable labels are well known in the art and include radioactive isotopes, e.g., ^{32}P, ^{3}H, ^{125}I, and ^{14}C, fluorescers, chemiluminescers, chromophores, and the like, as well as ligands such as biotin and haptens which, while not directly detectable, can be readily detected by reaction with labeled forms of their specific binding partners, e.g., avidin and antibodies, respectively.

(2) Detection by hybridization with labeled probe - This approach relies upon a further hybridization step for the detection of the RNA transcripts. A variety of methods are known for the preparation of probes which comprise labels that are directly or indirectly detectable. The labels can be any of the same materials mentioned immediately above.

(3) Detection by hybridization and use of anti-hybrid reagent - The detection probe can also be selected in a manner such that the hybrids formed with the RNA transcripts are unique in the mixture and thereby selectively detectable through the use of anti-hybrid reagents. A variety of anti-hybrid antibodies are known in the literature, including anti-DNA/RNA and anti-RNA/RNA antibodies (U.S. Pat. No. 4,833,084 and European Patent Publication No. 163,220), anti-DNA/DNA antibodies (U.S. Pat. No. 4,623,627), and antibodies to intercalation complexes (U.S. Pat. No. 4,563,417). The anti-hybrid antibody will advantageously be modified to contain a detectable label as above.

(4) Detection of RNA transcripts - The transcription products themselves can be detected in solution by separating RNA from the mixture, or by first destroying unreacted rNTPs with phosphatases, and then adding reagents that produce a detectable response to RNA, such as the bioluminescent system involving reaction with polynucleotide phosphorylase, pyruvate kinase and firefly luciferase (e.g., as described by C.P.H. Vary (1987) Nucleic Acids Res. 15:6883-6897).

Of course, since the transcription system can be designed to produce transcripts of a specified size, in such case they can be readily identified by size resolution methods such as gel electrophoresis.

The present invention will now be illustrated, but is not intended to be limited, by the following examples.

EXAMPLE 1

Ligation of probe to complementary oligonucleotide and transcription

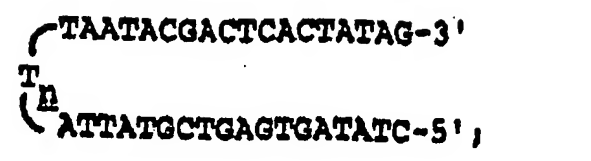
This example demonstrated that a sample sequence after hybridization and ligation to a probe of the present invention will provide RNA synthesis by transcription.

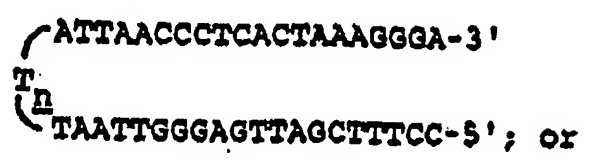
The oligonucleotides HNT and CNT shown below were synthesized in an Applied Biosystems (Foster City, CA, USA) Model 380 B oligonucleotide synthesizer using the supplier's reagents.

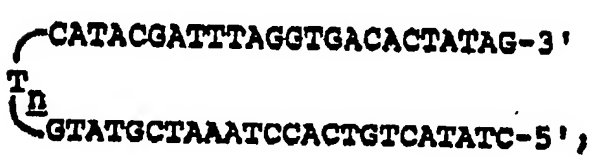
```
HNT:      ATTATGCTGAGTGATATC-5'
          (T)7
          TAATACGACTCACTATAGGGAGAAGTCTGCCGTTAC-3'

CNT:      3'-CCTCTTCAGACGGCAATG-5'
```

HNT, after gel electrophoretic purification was phosphorylated at its 5′ end using polynucleotide kinase according to the method described in Maniatis et al (1982) Molecular Cloning, Cold Spring Harbor, p. 122. The kinased HNT was then hybridized with CNT at 37°C for 5 minutes and then 100 units of T4 DNA ligase (Boehringer Mannheim, Indianapolis, IN, USA) and ATP (10 mM final concentration) were added. The ligation reaction was carried out by incubating the mixture at 37°C for 2 hours. HNT and CNT amounts

for T7,


for T3,


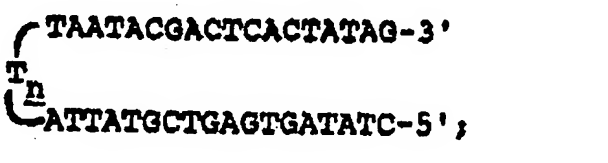
for SP6,


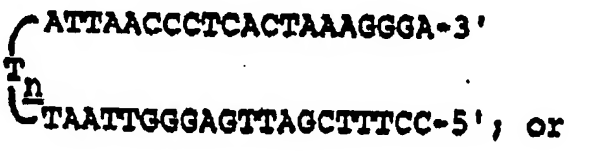
wherein n is an integer from 2 to 50 inclusive.

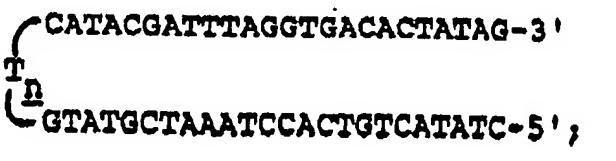
3. A method for amplifying a particular target nucleic acid sequence, comprising the steps of:
(a) hybridizing said target sequence with a probe of claim 1 in a liquid mixture,
(b) ligating the resulting hybridized target sequence to the 5′ end of said hairpin-forming self-complementary sequence,
(c) adding to the ligated hybrids said RNA polymerase and ribonucleoside triphosphates sufficient for transcription of said target sequence in the hybrids, and
(d) allowing the resulting transcription of said target sequence to take place for predetermined period of time to accumulate the resulting complementary RNA transcription product as an amplification of said target sequence.

4. method of claim 3 wherein the resulting RNA transcription product is further amplified in a second stage amplification.

5. The method of any of claims 3 and 4 wherein said polymerase is a DNA-dependent RNA-polymerase, preferably T7, T3, or SP6 bacteriophage RNA polymerase, and preferably wherein said promoter region comprises the following base sequence shown in its hairpin form:

for T7,


for T3,


for SP6,


wherein n is an integer from 2 to 50 inclusive.

6. A method for detecting a particular target nucleic acid sequence in a test sample, comprising the steps of:
(a) hybridizing said target sequence in the test sample with a probe of claim 1 in a liquid mixture,
(b) ligating the resulting hybridized target sequence to the 5′ end of said hairpin-forming self-

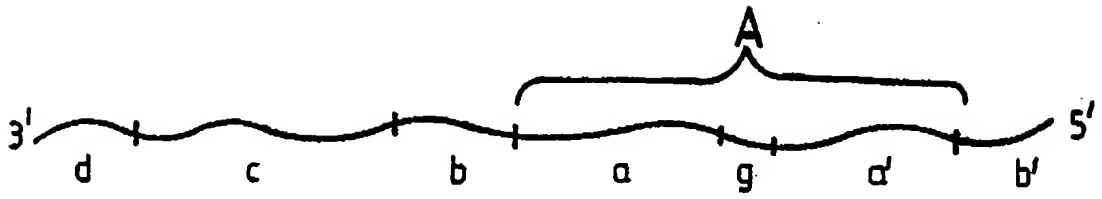


FIG. 1

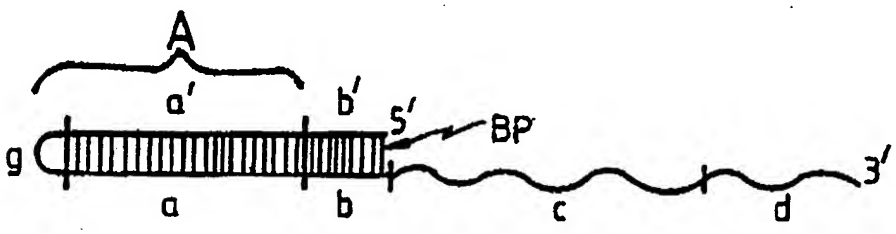


FIG. 2

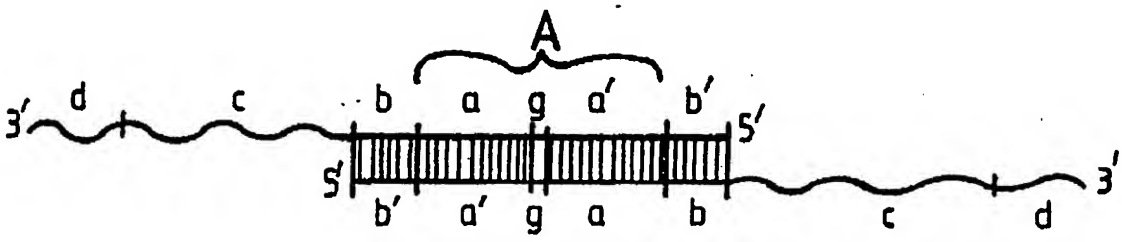


FIG. 3

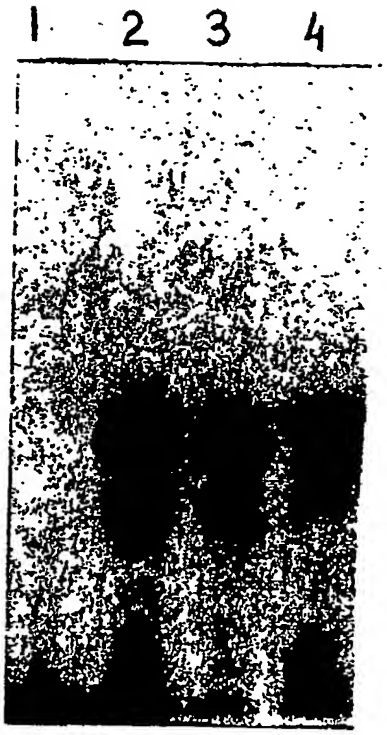


FIG. 5